



Factors Affecting Microbial Degradation of Polycyclic Aromatic Hydrocarbon Phenanthrene in the Caribbean Coastal Water¹

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Studies were conducted to assess factors that may influence the rate and extent of biodegradation of polycyclic aromatic hydrocarbons (PAHs) in waters of Guayanilla Bay (latitude, 18°N; longitude, 66.45°W) Puerto Rico. Phenanthrene was used as a model PAHs compound. Both the rate and extent of phenanthrene degradation by natural microbial flora present in seawater samples from Guayanilla Bay were quite slow. Addition of KNO₃ as a source of inorganic nitrogen (N) resulted in a 10-fold increase in the rate of phenanthrene degradation within a 125 h period, whereas, addition of K₂HPO₄ as a source of inorganic nutrient phosphorus (P) had no effect. Phenanthrene degradation was strongly inhibited when seawater pH was adjusted to 10.0. Phenanthrene in seawater samples degraded rapidly when first pretreated with hydrogen peroxide (H₂O₂) and then inoculated with a known indigenous phenanthrene degrading bacterium, *Alteromonas* sp. Pretreatment of phenanthrene with Triton-x-100 had little or no effect on its degradation by the same bacteria, whereas, degradation in samples preheated at 60°C was somewhat inhibited. © 1999 Elsevier Science Ltd. All rights reserved

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of environmentally persistent compounds, and some are considered to be environmental pollutants (Wise and Frahenhold, 1981). Considering their mutagenic and carcinogenic properties, 16 PAHs have been listed as priority pollutants by the United States Environmental Protection Agency (USEPA). In marine environments, most PAHs do not dissolve well in water and tend to accumulate in sediments (Means *et al.*, 1980). In coastal marine environments, sources of PAHs include atmospheric deposition, petrochemical industries, domestic and industrial wastewaters, rivers and spillage of petroleum products from ships. Concentrations of PAHs in marine sediments in urbanized estuaries may exceed 100,000 ng/g of sediments (Shiaris and Jambard-Sweet, 1986; Shiaris, 1989). As noted above, its presence in coastal environments may pose a potential threat to public health and marine life.

Coastal environments of Puerto Rico are prime repositories of PAHs because most industries as well as urban centers are located on the coast. Particularly, for over 20 yr, the Guayanilla coast was the site of one of the biggest concentrations of petrochemical industries in the world until these were shut down in 1982. Few studies have been done to determine the fate of pollutants at this site or the recovery of the coastal environment of Guayanilla following closure of the industrial complex.

PAHs are degraded primarily by biological transformation. Thus, microbial degradation represents a potential route for the elimination of PAHs from the environment. However, despite the abundance of information on degradation of PAHs by pure cultures (Cerniglia, 1984; Schocken and Gibson, 1984), the rate of biodegradation in coastal water and sediments has proven difficult to ascertain. Compared to pure cultures,

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PAHs degrade much more slowly in open waters and sediments, but physico-chemical factors may possibly influence the degradation of PAHs in natural environments. For example, several published reports, including our own, have indicated that in some environments, low concentrations of inorganic nutrients may adversely affect the rates of biodegradation of toxic chemicals (Manilal and Alexander, 1991; Jones and Alexander, 1991; Lewis and Hodson, 1986; Zaidi et al., 1996). Under anaerobic conditions, rates of degradation of PAHs may be negligible (Baur and Capone, 1985).

To devise successful and improved strategies for environmental clean-up of toxic organic compounds, it is critically important to understand those conditions under which toxic compounds can be removed quickly and effectively from the environment, thus minimizing adverse effects. The present study focuses on factors that may influence the rate and extent of biodegradation of PAHs in Guayanilla Bay waters. Phenanthrene was used as a model PAH compound to examine the effects of (1) added inorganic P and N, (2) glucose, (3) variable pH, (4) pretreatment of substrate with hydrogen peroxide, surfactant and (5) heat on biodegradation.

Materials and Methods

Phenanthrene (>96% purity), agarose type VII (low gelling temperature) and cycloheximide were obtained from Sigma Chemical St. Louis, MO, and Noble agar from Difco Laboratories, Detroit, MI.

A bacterial strain capable of degrading phenanthrene previously had been isolated from Guayanilla coastal water and identified as an *Alteromonas* sp. Cultures were deposited at the Northern Regional Research Laboratory culture collection and assigned reference number NRRL P2030 (Zaidi et al., 1999). Another bacterium (strain M) was isolated from Mayaguez coastal water. Seawater and sediment samples were collected from Guayanilla Bay and stored in a refrigerator and used within 2–3 weeks after collection.

Biodegradation of phenanthrene was determined by measuring CO_2 production in a fully computerized closed circuit Micro-Oxymax respirometer system (Columbus Instrument International Corporation, Columbus, OH) equipped with expansion interfaces, condensers and a temperature-regulated water bath. All tests and controls were run in duplicate in 250 ml respirometer chambers. Sample chambers were placed in the water bath at a constant temperature of 25°C and tests conducted in either 20 ml sterile or non-sterile seawater containing phenanthrene (10 µg/ml) and the selected isolate as indicated. Sample chambers were automatically refreshed and the total accumulation of CO_2 resulting from biodegradation recorded at suitable intervals. Aerobic biodegradation results in the production of CO_2 . Earlier studies have indicated a positive correlation between the amount of CO_2 released and the amount of degradation of the substrate (Zaidi et al.,

1988); the level of CO_2 measured by respirometry is a good indicator of both the extent and rate of degradation.

The effects of inorganic nitrogen (N) and phosphorus (P) on phenanthrene degradation were measured in duplicate samples, of sterile and non-sterile seawater samples amended with 10 µg/ml of phenanthrene, to which either 100 µg/ml of KNO_3 or K_2HPO_4 were added. Degradation was evaluated both in seawater with naturally occurring indigenous microbial flora, as well as by an isolated strain of *Alteromonas* sp. described earlier. In some samples, glucose (100 µg/ml) was added to the filter-sterilized Guayanilla Bay water along with phenanthrene (10 µg/ml) to assess the effect of glucose on the degradation by *Alteromonas* sp.

The effect of pH (6.0, 8.0, or 10.0) and 0.001% of an anionic detergent Triton-x-100, on phenanthrene degradation by the indigenous microbial flora was investigated in seawater samples containing phenanthrene.

To assess the effect of pretreatment of substrate on its degradation, phenanthrene was treated either with 1% hydrogen peroxide, or by heating at 60°C for 5 min; sterile seawater samples containing phenanthrene were first treated as indicated prior to adding the bacterium.

Results

When phenanthrene was added (10 µg/ml) to seawater samples from Guayanilla Bay, both the rate and extent of phenanthrene degradation by natural microbial flora present in these samples were quite slow (Fig. 1). Addition of KNO_3 (100 µg/ml) samples as a source of inorganic nitrogen (N) resulted in a 10-fold increase in the rate of phenanthrene degradation within 125 h, as

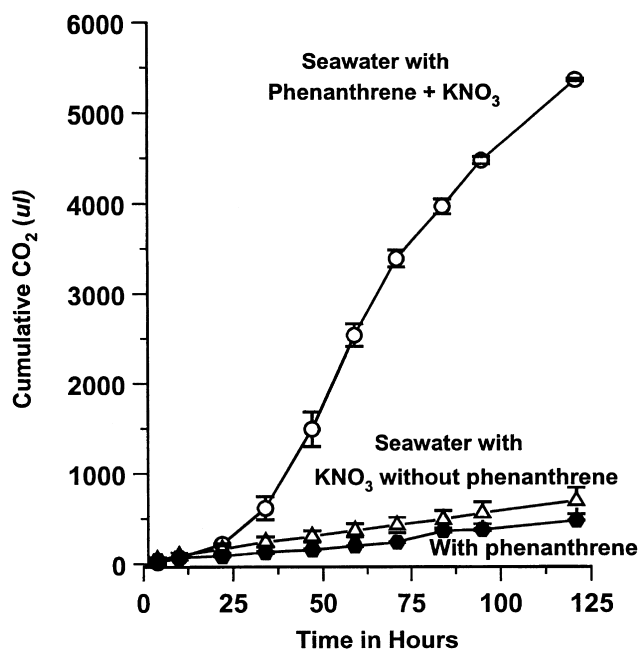


Fig. 1 Effects of inorganic N (10 µg/ml) on biodegradation of 10 µg/ml of phenanthrene in Guayanilla Bay water.

evidenced by the total accumulated CO_2 volume (Fig. 1). Control seawater samples containing either phenanthrene or KNO_3 alone showed no increase in CO_2 levels, indicating that the observed increase in biodegradation was due to the added inorganic nitrogen. Also, increasing concentrations of KNO_3 from 200 to 300 $\mu\text{g}/\text{ml}$ did not further enhance phenanthrene degradation (data not shown). On the other hand, addition of K_2HPO_4 (100 $\mu\text{g}/\text{ml}$) as a source of inorganic nutrient phosphorus (P) had no effect on phenanthrene degradation by the indigenous microbial flora in the water samples. Samples with K_2HPO_4 alone showed degradation rates similar to samples with added phenanthrene (Fig. 2). Samples with added KNO_3 were used as a positive control. Altering the pH of seawater from neutral to pH 6.0 and pH 8.0 had very little or no effect on phenanthrene degradation. However, at pH 10.0, the effect was more dramatic where phenanthrene degradation was markedly reduced (Fig. 3).

Sterile seawater samples from Guayanilla Bay supplemented with phenanthrene pretreated with 1% hydrogen peroxide, and an inoculum of an indigenous phenanthrene degrading bacterium, *Alteromonas* sp. (NRRL P2030), enhanced considerably the rate of phenanthrene degradation (Fig. 4). The production of CO_2 increased some 16-fold within 10 h relative to controls without any pretreatment (Fig. 4). These findings were also confirmed by comparison with another phenanthrene degrading bacterium, strain M, obtained from Mayaguez coastal water (data not shown).

Sterile seawater samples containing phenanthrene were heated to 60°C to increase the solubility of phenanthrene. *Alteromonas* sp. was then added to both experimental and controls (unheated) samples. Surprisingly, phenanthrene degradation was inhibited in pre-heated samples (Fig. 5). Also, pretreatment of phenanthrene by 0.001% Triton-x-100 had little or no effect on its degradation in seawater samples by indigenous flora. The rapid degradation of phenanthrene by indigenous microorganisms in the presence of a surfactant and KNO_3 indicated that Triton-x-100 was not toxic to the microbial population present in the seawater (Fig. 6).

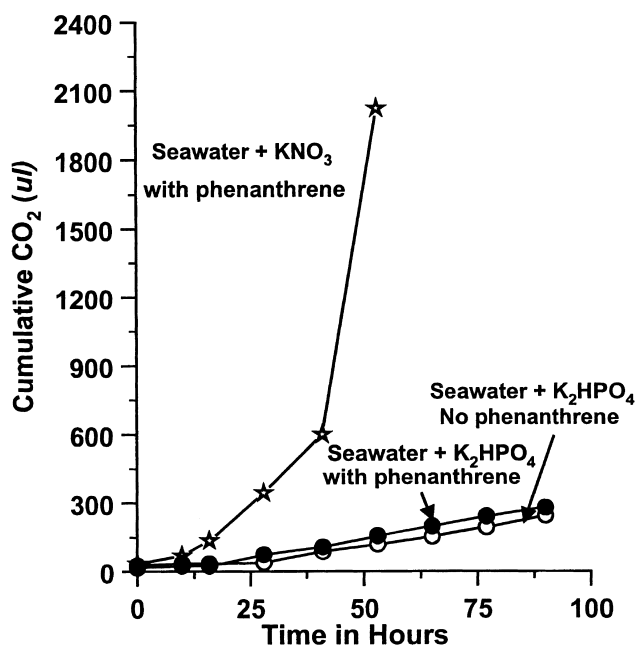


Fig. 2 Effects of inorganic P (10 $\mu\text{g}/\text{ml}$) on the biodegradation of 10 $\mu\text{g}/\text{ml}$ of phenanthrene in Guayanilla Bay water.

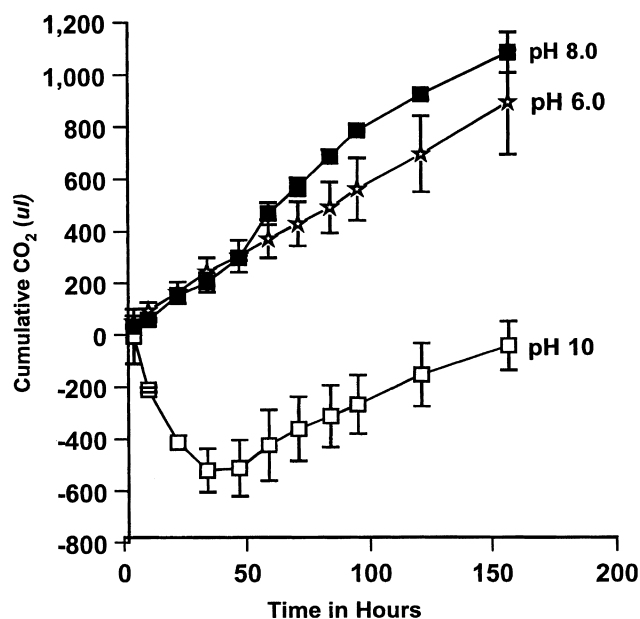


Fig. 3 Effects of pH on the degradation of 10 $\mu\text{g}/\text{ml}$ of phenanthrene in the Guayanilla Bay water.

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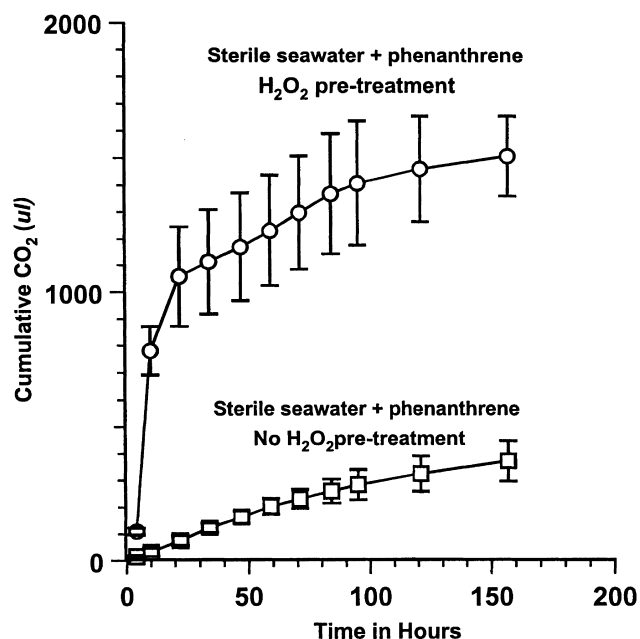


Fig. 4 Effects of H_2O_2 (1%) pretreatment on the degradation of 10 $\mu\text{g}/\text{ml}$ of phenanthrene in sterile Guayanilla Bay water by *Alteromonas* sp.

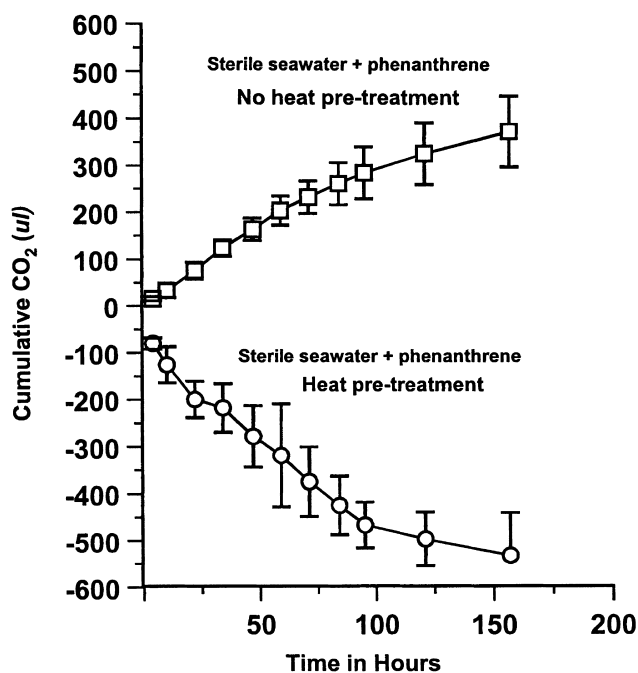


Fig. 5 Effect of heat pretreatment on the degradation of 10 µg/ml of phenanthrene by *Alteromonas* sp. in sterile Guayanilla Bay water.

Addition of glucose (100 µg/ml) to seawater samples alone resulted in elevated levels of CO₂ production. Further addition of phenanthrene showed only slightly enhanced degradation of phenanthrene (Fig. 7).

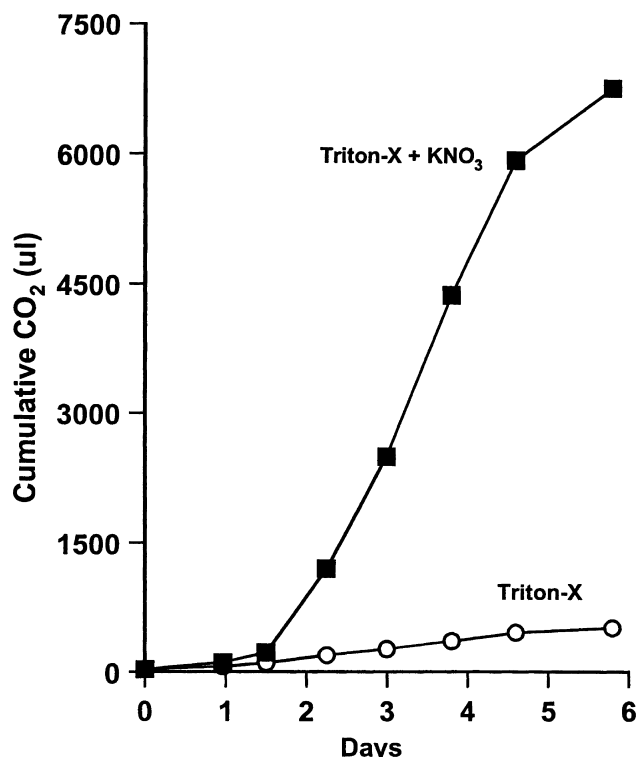


Fig. 6 Effect of Triton-x-100 on the degradation of 10 µg/ml of phenanthrene with and without N (100 µg/ml).

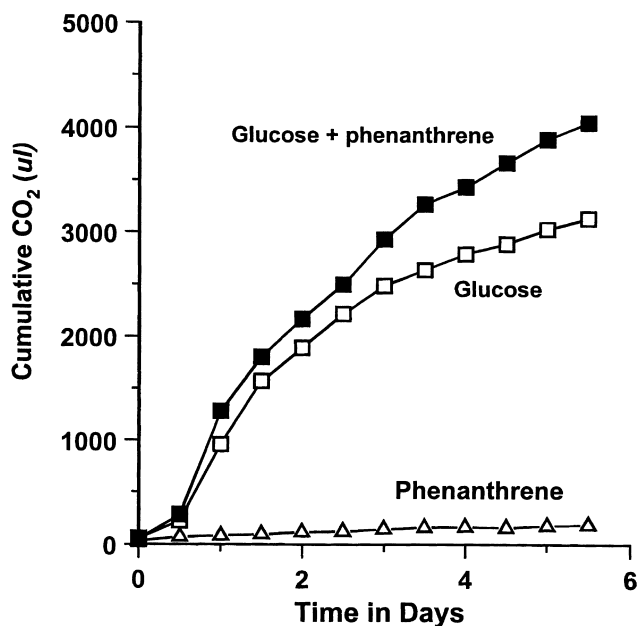


Fig. 7 Effect of glucose (100 µg/ml) on the degradation of phenanthrene by *Alteromonas* sp. in sterile Guayanilla Bay water.

Discussion

The transformation of PAH compounds in the environment is mainly through microbial processes, but is also influenced by a number of environmental factors (Manilal and Alexander 1991). Efforts to remove PAHs from coastal environments by microbial activity would require knowledge of the concentration of the toxic compounds and the rate at which the compound will be degraded. Equally important would be to understand how environmental factors would influence the degradation process. The major limitation in bioremediation of hydrocarbon-contaminated soil and water is the availability of nutrients, such as nitrogen and phosphorus (Rosenburg *et al.*, 1992). The degradation of phenanthrene in seawater treated with nitrate was quite interesting. It has been shown in an earlier study (Atlas, 1981) that the addition of a fertilizer containing nitrogen and phosphorus stimulated hydrocarbon degradation in areas contaminated by crude oil. Similarly, work done in our laboratory (Zaidi *et al.*, 1988a) has indicated that phosphorus but not nitrogen was a limiting factor in the biodegradation of *p*-nitrophenol (PNP) in Lake water. Data from the present study, however, indicated that in the Guayanilla Bay waters, the nutrient P did not appear to be a limiting factor for phenanthrene degradation. Availability of an easily used carbon source glucose also did not enhance degradation of phenanthrene in Bay water, indicating that carbon is not a limiting factor. Also, the lack of N may slow down the biodegradation of phenanthrene and cause it to accumulate in the sediments, where nutrients are not a limiting factor. This is in fact the case, as evidenced from our experiments conducted with waters collected just above sediments,

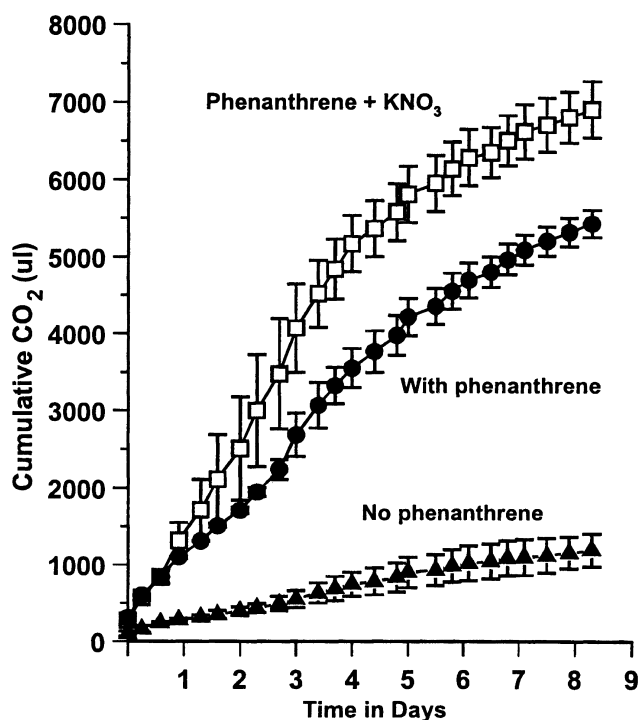


Fig. 8 Effects of inorganic N (10 $\mu\text{g}/\text{ml}$) of phenanthrene in Guayanilla Bay sediments.

indicating that the addition of nitrate only slightly enhanced degradation of phenanthrene (Fig. 8).

A marked reduction in phenanthrene degradation at an elevated pH was achieved using Bay water. Guayanilla, located in the south of Puerto Rico is dry and hot most of the year, receiving little rainfall. Therefore, any fluctuation in pH higher than 8.0 may potentially slow down the degradation of PAHs in the Bay waters. Furthermore, lack of available nitrogen combined with high pH in Bay water may further increase the residence time of PAHs in the Bay water.

Increased rate of phenanthrene degradation after H_2O_2 treatment was quite dramatic. Hydrogen peroxide, though a weak oxidant, can in alkaline medium readily cleave C–C bonds of carbohydrates in the presence of metal ions (Blattner and Ferrier, 1985). This reaction has been applied in the production of oxidized starches and other modified carbohydrates (Wing, 1994; Parovuori *et al.*, 1995). Seawater contains most of the requisite cations and pH range for the free radical process involved in a hydrogen peroxide reaction. A plausible explanation for the experimental observation could be that cleavage of dihydroxylated aromatic ring is the slow step of phenanthrene catabolism under normal conditions. The presence of H_2O_2 at this point, on the other hand, shifts the reaction equilibrium position far to the right, providing the observed degradation, as evidenced by the measured exponential increase in CO_2 evolution. Further research on intermediate products formed during biodegradation of phenanthrene is required to address more adequately the role of H_2O_2 .

Bacteria can better use phenanthrene when it is in the dissolved state (Wodzinski and Coyle, 1974). However, phenanthrene is relatively insoluble in water (Banerjee, 1985), but its solubility has shown to increase in the presence of non-ionic surfactants (Grimberg *et al.*, 1995). On the other hand, its solubility further decreases with increasing salt concentration that are typical of marine environments (Whitehouse, 1985) and this may very well be the reason as to why pretreatment of phenanthrene with Triton-x-100 had little or no effect on its biodegradation in our experiments. In this regard, it is worth noting that the effects of addition of non-ionic surfactants on microbial degradation of hydrophobic organic compounds have been described to vary from beneficial, to detrimental, to ineffective. For example, Triton-x-100 completely prevented the mineralization of hexadecane dissolved in heptamethylnonane (Efroymson and Alexander, 1991), but increased both the rate and extent of mineralization of naphthalene dissolved in the same solvent (Efroymson and Alexander, 1991). Surfactants can also prevent the adherence of cells to organic-aqueous interface affecting bacterial degradation of substrate (Aiba *et al.*, 1969). In recent years, surfactants have also been used to disperse oils, after spills in marine waters, but further studies are warranted to understand the benefits of its use and the exact role surfactants play in the biodegradation process.

The present study yielded important information on the fate of PAHs degradation in coastal water. Particularly, enhanced degradation due to the added inorganic nitrogen and H_2O_2 pretreatment, or inhibition of degradation at higher pH, all provided fundamental knowledge that would be useful in developing and improving environmental clean-up strategies for PAHs.

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